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Inactivation of luminous *Vibrio* spp. by free chlorine

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Abstract
In vitro inactivation of penaeid shrimp larval pathogens, *Vibrio harveyi* and *V. splendidus* biovar 1, by free chlorine and the influence of organic matter on the bactericidal activity of chlorine were assessed. More than 5 log unit (>99.99%) reduction in luminous bacteria from ≥ log 6.00/ml within the first 60 sec of exposure to free chlorine at 1 ppm level was observed. Chlorine was ineffective at <50 ppm levels to inhibit luminous *Vibrio* spp in the presence of 0.1% peptone as interfering organic agent. These results revealed that luminous bacteria are highly susceptible to chlorine but the bactericidal activity of chlorine is affected by organic substance.
Key words: *Vibrio* spp., Chlorine

Introduction

Hatchery production and rearing of penaeid shrimp larvae require good quality water (Lavilla-Pitogo *et al.* 1990). Water treatment in shrimp hatcheries usually consists of sedimentation and filtration. However, in cases where microbial contamination is a problem, such physical treatment is inadequate and some form of disinfectant is needed. Chlorine is a powerful disinfectant that has long been used to control microorganisms in water. Laboratory and field studies demonstrated the biocidal effect of chlorine on viral (LeBlanc and Overstreet 1991) and bacterial pathogens (Sako *et al.* 1988, Pascho *et al.* 1995) of aquaculture importance. Vibriosis caused by luminous *Vibrio* spp., particularly *Vibrio harveyi*, is a problem to shrimp hatchery operations globally (Lavilla-Pitogo *et al.* 1990, Karunasagar *et al.* 1994, Mohney *et al.* 1994, Abraham *et al.* 1997a). The purpose of this work was to examine the *in vitro* inactivation of penaeid shrimp larval pathogens such as *V. harveyi* and *V. splendidus* biovar 1 by chlorine and the influence of organic matter on the bactericidal activity of chlorine under laboratory condition.

Materials and methods

Test organisms

Two species of luminous bacteria *viz.*, *Vibrio harveyi* SW_{57} and *V. splendidus* biovar 1 SW_1, isolated from penaeid shrimp hatchery source water (Abraham *et al.* 1997b) were used.

Chlorine solution

A commercial preparation of sodium hypochlorite containing available chlorine concentration of 20 mg/ml, as determined by the standard iodometric titration method (APHA/AWWA/WEF 1995), was used. This solution was diluted in seawater (salinity 35 ppt), aged for more than 3 months and filtered, to provide 1-100 ppm free chlorine (target) concentrations when mixed with the inoculum.

Interfering substance

This was used to test the influence of organic substance on the efficacy of target chlorine levels. Peptone at 0.1% (w/v), dissolved in aged seawater, was used as interfering substance. The medium was adjusted to pH 7.8 and sterilized at 121°C for 15 min. Half strength seawater made from 35 ppt aged seawater was used as diluent.

Neutralizing solution

A 0.01 mol/l sodium thiosulphate solution prepared in half strength-aged seawater was the neutralizing solution.

Culture medium and preparation of cell suspensions

Complex seawater (CSW) medium, with or without 1.5% agar, containing 75% aged seawater, 25% distilled water, 0.5% peptone, 0.3% yeast extract and 0.3% glycerol was used for the growth and maintenance of luminous *Vibrio* spp. The pH of the medium was 7.80. Cell suspensions of *V. harveyi* and *V. splendidus* biovar 1 was prepared separately as described in Abraham *et al.* (1997a).

Susceptibility of luminous bacteria to free chlorine

These experiments were designed to reproduce luminous bacterial inactivation, but not intended to simulate natural environments. Luminous bacterial inactivation was done in Erlenmeyer flasks containing 100 ml of sterile aged seawater (pH 8.0). Sodium hypochlorite solution was added to these flasks to get a concentration of 1, 5 and 10 ppm of free chlorine separately. The flasks were then inoculated with *V. harveyi* SW_{57} and/or *V. splendidus* biovar 1 SW_1 at $10^6 - 10^7$ cells/ml levels and incubated at 30±1°C. The numbers of bacteria in each flask were determined after 1 min, 30 min and 24 h of exposure to chlorine and 24 h after neutralization. The effect of neutralizing agent, 0.01

mol/l sodium thiosulphate in half strength seawater, on luminous bacteria was evaluated by incubating the cells in neutralizing solution for 30 min. The numbers of bacteria before and after exposure to neutralizing agent were determined.

Enumeration of luminous bacterial counts (LBC) was done by spread plating on CSW agar and/or by 5 tube most probable number (MPN) technique using CSW broth. Before enumeration, the residual chlorine present in one ml each of the samples drawn from chlorine treated flasks were neutralized by vigorous agitation in sterile 9 ml of 0.01 mol/l sodium thiosulphate solution (10^{-1} dilution). Subsequent ten fold serial dilutions were made in sterile half strength seawater. All the plates and tubes, after inoculation, were incubated at 30±1°C for 24-72 h. Aliquots from CSW broth were streaked on to CSW agar and incubated for 48 h at 30±1°C to record MPN value. Bacterial numbers were recorded as counts/ml. All experiments were repeated at least 3 times and the percentage survival calculated.

Results and discussion

Aquaculture is a large consumer of chlorine products. The use of chlorine has been recommended to eliminate shrimp pathogens in hatcheries (Baticodos and Pitogo 1990, LeBlanc and Overstreet 1991, Lewis *et al.* 1992) and as a disinfectant and sanitary agent for fish tanks, raceways, utensils, contaminated equipments and effluents at 200 mg/l level (LeBlanc and Overstreet 1990, Pascho *et al.* 1995). The results presented in Table 1 showed that both *V. harveyi* and *V. splendidus* biovar 1 reacted in a more or less identical way to the bactericidal effect of chlorine. A reduction of more than 5 log unit (>99.99%) from >log 6.00/ml was achieved within the first 60 sec of exposure to free chlorine at 1 ppm level in the absence of any interfering agent, which indicated that luminous bacteria are highly susceptible to free chlorine. At 1 and 5 ppm levels, luminous bacterial populations were completely eliminated and no recovery was possible even after neutralization with 0.01 mol/l sodium thiosulphate and enrichment in CSW broth. Similar chlorine effect was reported for luminous bacteria (Baticados and Pitogo 1990), *V. anguillarum* and *V. ordalii* (Sako *et al.* 1988), *Renibacterium salmoninarum* (Pascho *et al.* 1995). The mechanisms of chlorine inactivation vary among microorganisms and are a result of general oxidation of reduced chemical species. The populations of *V. harveyi* (log 6.778/ml) and *V. splendidus* biovar 1 (log 6.873/ml) exposed to thiosulphate neutralizer for up to 30 min failed to show any difference from the respective counts of log 6.781/ml and log 6.872/ml determined before exposure. The neutralizer was, therefore, assumed to exert no effect on results.

Table 1. Effect of sodium hypochlorite on the growth (counts/ml) of *Vibrio harveyi* and *V. splendidus* biovar 1 in sterile seawater

Treatment / Exposure time	*Vibrio harveyi* SW_{57}		*V. splendidus* biovar 1 SW_1	
	1 ppm	5 ppm	1 ppm	5 ppm
Before chlorination	2.50×10^6	4.45×10^6	2.20×10^6	2.80×10^6
After chlorination				
1 min	1.00×10^1	$<1.00 \times 10^1$	1.00×10^1	<1.00
30 min	<1.00	<1.00	<1.00	<1.00
24 h	<1.00	<1.00	<1.00	<1.00
After neutralization				
24 h	<1.00	<1.00	<1.00	<1.00

Results on the interference of organic substance on the bactericidal activity of free chlorine, as shown in Table 2, revealed that chlorine is ineffective at <50 ppm level to inhibit luminous bacteria (*V. harveyi* and *V. splendidus* biovar 1) in the presence of 0.1% peptone. No bactericidal effect was seen at 1 ppm level. At 5-20 ppm levels, the LBC reduced slightly immediately after chlorination and then increased to ≥ log 8.00 cells/ml in 24 h. Chlorine effect was apparent at 50 ppm level. Chlorination and oxidation reaction between amino group and chlorine are most likely responsible for this effect. These results corroborate with the findings of earlier studies (Sae-Oui *et al.* 1987, Chanratchakool 1995). According to Chanratchakool (1995), the minimum effective concentration of hypochlorite to inhibit *V. harveyi* and other *Vibrio* spp was 2-8 ppm and 2-16 ppm of active chlorine, respectively. About 16 ppm of active chlorine was required for disinfection of water from the shrimp farm containing > log 4.00 organisms/ml. Sae-Oui *et al.* (1987), however, reported that *V. harveyi* could be completely killed by treating with calcium hypochlorite at 20-30 ppm. Nevertheless, Karunasagar *et al.* (1995) using microcosm experiments demonstrated that chlorination would not kill *V. harveyi* present in sediments and, therefore, repopulation of the system occurs immediately after dechlorination. The results of the present *in vitro* study also confirmed that chlorine treatment would not eliminate shrimp larval pathogens such as *V. harveyi* and *V. splendidus* biovar 1 in a system where particulate and suspended organics are present.

Table 2. Interference of 0.1% peptone on bactericidal effect of sodium hypochlorite: effect on luminous bacteria

Treatment / Exposure time	Counts / ml					
	1ppm	5ppm	10ppm	20ppm	50ppm	100ppm
Vibrio harveyi SW_{57}						
Before chlorination	$5.60x10^6$	$7.80x10^6$	$9.00x10^6$	$6.00x10^6$	$4.75x10^6$	$5.20x10^6$
After chlorination						
1min	$5.60x10^6$	$6.90x10^6$	$8.00x10^6$	$2.80x10^6$	$1.50x10^1$	<1.00
30 min	$5.65x10^6$	$7.30x10^6$	$8.30x10^6$	$1.30x10^4$	<1.00	<1.00
Vibrio splendidus biovar 1 SW_1						
Before chlorination	$4.30x10^6$	$4.70x10^6$	$7.50x10^6$	$6.10x10^6$	$8.00x10^6$	$5.00x10^6$
After chlorination						
1min	$4.25x10^6$	$3.45x10^6$	$4.50x10^6$	$1.50x10^6$	$1.00x10^1$	<1.00
30 min	$4.30x10^6$	$3.55x10^6$	$4.65x10^6$	$6.10x10^3$	<1.00	<1.00

The counts of *Vibrio harveyi* SW_{57} and *V. splendidus* biovar 1 SW_1 were increased after 30 min in 1,5,10 and 20 ppm levels. No growth of luminous bacteria was seen in 50 and 100 ppm levels even after 24 h.

It is worth mentioning here that sodium hypochlorite was demonstrated to be effective at relatively high concentrations, ≥50 ppm, and for longer period of time for the control of baculovirus in shrimp mariculture facilities (Lewis *et al.* 1992). In shrimp grow-out systems, chlorine disinfection is being followed in the reservoirs, although with variable success, to inactivate the causative agent of white spot viral disease and other bacterial pathogens. As majority of the components of source water of the shrimp aquaculture systems are particulate and suspended organics, these components must, therefore, be thoroughly removed by mechanical filtration or by sedimentation for effective hatchery disinfection protocol.

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